

Review

Smad4-mediated TGF- β signaling in tumorigenesis

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Abstract

The dual effects of TGF- β signaling on tumor initiation and progression are cell-specific and yet to be determined under distinct contexts. A number of genetically manipulated mouse models with alterations in the TGF- β pathway genes, particularly the pivotal *Smad4*, revealed that these genes play crucial functions in maintaining tissue homeostasis and suppressing tumorigenesis. Loss of *Smad4* plays a causal role in initiating squamous cell carcinomas of skin and upper digestive tract as well as adenocarcinomas of gastrointestinal tract. However, for some cancers like pancreatic and cholangiocellular carcinomas, *Smad4* deficiency does not initiate the tumorigenesis but acts as a promoter to accelerate or synergize the development and progression of cancers that are started by other oncogenic pathways. Intriguingly, emerging evidences from mouse models have highlighted the important roles of non-cell autonomous effects of *Smad4*-mediated TGF- β signaling in the inhibition of oncogenesis. All these data have greatly deepened our understanding of molecular mechanisms of cell-autonomous and non-cell autonomous effect of *Smad4*-mediated TGF- β signaling in suppressing carcinogenesis, which may facilitate the development of successful therapies targeting TGF- β signaling for the treatment of human cancers.

Key words: TGF- β , *Smad4*, mouse model, tumorigenesis

Introduction

The tumorigenesis of all human cancers can be divided into a series of landmarks that are required to be overcome by a “cancer cell.” First, the cells within a tissue undergo genetic or epigenetic alterations and acquire the potential to become malignant, whereby they undergo unregulated proliferation and recruit a blood supply, and finally, the cells invade and metastasize to other sites [1]. However, this is not a very favorable course for oncogenic cells because in addition to known cancer defense mechanisms such as DNA repair, there exists a dynamic and reciprocal struggle between the genetically altered cells and their microenvironment. Malignant cells must sub-

vert the microenvironmental controls for survival; however, the tumor microenvironment, which includes extracellular matrix, blood vasculature, inflammatory cells, and fibroblasts, hinders the tumor development by the virtue of a network of soluble growth factors and cytokines within the stroma, which act in an autocrine and paracrine fashion. Any defection by the microenvironment during the anti-cancer battle may damage the equilibrium and result in a spectrum of dysfunctions, including cancer [2].

Among the pathways involving growth factors that serve as the mediators of tumorigenesis, the transforming growth factor- β (TGF- β) signaling

pathway has attracted much attention [3]. TGF- β plays a confirmed yet complicated role in directing the autonomous, local, and systemic cellular responses that together regulate the initiation, progression, and prognostic outcome of human cancers [4,5]. Other pathways altered in human cancer might contribute to the TGF- β -mediated regulation of tumorigenesis to some extent [6-13]. Unlike fibroblast growth factor, insulin-like growth factor, and epithelial growth factor, which mainly act as tumor promoters by influencing cell proliferation, TGF- β plays a dual role in tumorigenesis. During initiation and early progression of the tumor, TGF- β serves as a tumor suppressor by inhibiting proliferation and accelerating apoptosis, which is supported by the fact that loss or mutation of the members of the TGF- β signaling pathway in humans causes unregulated cell growth and eventually cancer. In late stages of tumor progression, elevated levels of TGF- β promote tumor formation by facilitating migration, invasion, angiogenesis, and evasion of the immune system, with its increased production being associated with poor prognosis for patients [3]. However, the “double-edged sword” of TGF- β exerts both cell-specific and context-dependent effects. For example, TGF- β not only inhibits the uncontrolled proliferation of epithelial, endothelial and hematopoietic cells, but also mediates tumor promotion predominately through the surrounding stroma other than the pre-cancerous epithelial cells themselves [14]. Therefore, there is an urgent need to evaluate the mechanisms by which cell-specific and context-dependent responsiveness to TGF- β occurs in the fields of receptor ex-

pression, availability of downstream components, and establishment of crosstalk communication with other pathways.

Smads are the key intracellular mediators of transcriptional responses to TGF- β . In mammals, the 8 Smads are subdivided into 3 distinct classes: receptor-regulated Smads (R-Smads) comprising Smads 2 and 3 (transduce TGF- β signaling) and Smads 1, 5, and 8 (transduce bone morphogenetic protein (BMP) signaling); a common Smad called co-Smad4; and 2 inhibitory Smads (I-Smads), namely, Smads 6 and 7 [15]. Smad4 is the pivotal factor of the TGF- β pathway and functions as a key tumor suppressor. The germline mutation of *Smad4* gene causes Juvenile Polyposis Syndrome (JPS). Homozygous deletion or intragenic mutation of somatic *Smad4* gene frequently occurs in the carcinomas of the pancreas, gastrointestinal, and skin. Dysregulated Smad4 expression is also usually found in few types of cancers [16,17]. The gene knockout and transgenic techniques of genetic manipulation have been used to generate a number of mouse models that faithfully recapitulate the initiation and progression processes of human cancers and deepen our understanding of molecular mechanisms of cancer physiopathology. The development of a conditional knockout mouse bearing a floxed *Smad4* allele [18] and a spectrum of appropriate tissue-specific Cre transgenic mice have been used to elaborate the function and the related molecular mechanisms of Smad4-mediated TGF- β signaling in maintaining tissue homeostasis and suppressing tumorigenesis (see Table 1).

Table 1. *Smad4*-deficient mouse models that recapitulate human tumorigenesis

Tumor type	<i>Smad4</i> -deficient cells	Phenotypic cells	With combined mutations
<i>Smad4</i> complete knockout mice			
Tumors throughout the gastrointestinal tract	All type of cells	Gastrointestinal epithelial cells	Alone [51, 53] or with <i>Apc</i> ^{+/-} [50, 64, 65], <i>elf</i> ^{+/-} [58-60]
Tissue-specific <i>Smad4</i> conditional knockout mice			
Tumors throughout the gastrointestinal tract	T cells	Gastrointestinal epithelial cells	Alone [107]
Pancreatic ductal adenocarcinomas	Pancreatic progenitor cells	Pancreatic ductal epithelial cells	With <i>Kras</i> ^{G12D} [29, 31, 32] or <i>Kras</i> ^{G12D} ; <i>Ink4a</i> / <i>Arf</i> ^{Co/Co} [29]
Skin squamous cell carcinomas	Keratinocytes	Keratinocytes	Alone [79, 80] or with <i>Pten</i> ^{Co/Co} [80]
Head and neck squamous cell carcinoma	Oral epithelial cells	Oral epithelial cells	Alone [82]
Esophagus and forestomach Squamous Cell Carcinoma	Esophageal and forestomach epithelial cells	Esophageal and forestomach epithelial cells	Alone or with <i>Pten</i> ^{Co/Co} [81]
Cholangiocellular carcinoma	Hepatocytes and bile duct epithelial cells	Bile duct epithelial cells	With <i>Pten</i> ^{Co/Co} [101]
Breast squamous cell carcinoma	Mammary epithelial cells	Mammary epithelial cells	Alone [102]
Keratocystic odontogenic tumors	Odontoblasts or HERS cells	Odontoblasts/HERS cells or HERS cells	Alone [108]

Cell-autonomous effect of Smad4-mediated TGF- β signaling in suppressing tumorigenesis

Pancreatic cancer

Genetic dysregulation of TGF- β signaling pathway is commonly observed in pancreatic cancer [19]. The alternative name of human SMAD4, i.e., DPC4 (*deleted in pancreatic carcinoma, locus 4*), suggests the close relationship of loss of this gene with pancreatic cancer [20]. Several evidences support the role of SMAD4 as a tumor suppressor gene in pancreatic tumorigenesis. Loss of heterozygosity (LOH) at 18q, where SMAD4 gene is located, occurs in 90% of pancreatic carcinomas [21]. Homozygous deletion or intragenic inactivating mutations of SMAD4 gene as well as the complete loss of SMAD4 protein expression are observed in 50% ductal adenocarcinomas [20], 34% invasive adenocarcinoma of the Vater ampulla [22], and 55% endocrine pancreatic carcinomas [23]. The expression level of SMAD4 protein is inversely associated with histopathological grades of pancreatic cancers [24]. Loss of SMAD4 expression has also been postulated as the indication of pancreatic origin in metastatic carcinoma [19]. However, some studies have also suggested that compromised TGF- β signaling may account for the progression of pancreatic cancer rather than the initiation step. Restoration of SMAD4 in a variety of SMAD4-null pancreatic tumor cell lines did not affect proliferation but inhibited pancreatic tumor invasion and angiogenesis [25]. However, the role of Smad4-mediated TGF- β signaling in pancreatic cancer progression and metastasis is controversial. For instance, high expression of TGF- β isoforms in human pancreatic ductal adenocarcinoma tissues correlates with the poor prognosis [26]. Patients expressing SMAD4 unexpectedly exhibit significantly worse outcomes and did not benefit from surgery [27]. One study showed that cells expressing SMAD4 showed an enhanced TGF- β -mediated epithelial-to-mesenchymal transition (EMT) [28,29]. These instances highlighted the tumor promoting role of SMAD4 in pancreatic carcinogenesis.

Recently, the dual role of Smad4 was established in a cohort of mouse models of human pancreatic cancer. Selective *Smad4* or TGF- β type II receptor (*Tgfb β 2*) deletion in pancreatic epithelium had no detectable effect on pancreatic development or physiology, indicating an inculpable role of *Smad4* deficiency in initiating pancreatic tumorigenesis. However, when combined with activated Kras expression in mice, *Smad4* haploinsufficiency, loss of *Smad4* or

loss of *Tgfb β 2* accelerated the progression of Kras-initiated neoplasms to high-grade tumors. These *in vivo* results favor the conclusion that Smad4 mediates the tumor inhibitory action of TGF- β signaling, predominantly at the progressive stage of tumorigenesis [29-31]. *Smad4* deficiency also markedly induces the development of tumors into adenocarcinomas in the event of *Ink4a/Arf* loss and Kras activation. Interestingly, however, the adenocarcinomas in *Pdx1-Cre;Kras^{G12D};Smad4^{Co/Co};Ink4a/Arf^{Co/Co}* and *Pdx1-Cre;Kras^{G12D};Smad4^{Co/Co};Ink4a/Arf^{Co/+}* mice exhibited greatly reduced proportion of sarcomatoid histology which is commonly presented in those of *Pdx1-Cre;Kras^{G12D};Ink4a/Arf^{Co/Co}* and *Pdx1-Cre;Kras^{G12D};Ink4a/Arf^{Co/+}* mice, while maintaining a differentiated histopathology [29,32]. This finding validates the observations that intact Smad4 facilitates EMT and TGF- β -dependent metastasis in human pancreatic cancers [33,34]. Although the above studies have not addressed the conundrum of the Smad4 switch from a tumor-suppressive to a tumor-promotion pathway in pancreatic cancer, Smad4-dependent inhibition of β -catenin degradation [35] and the activation of signal transducers and activators of transcription 3 (Stat3) [28] as well as the effects of stromal fibroblasts [36-38] may be involved. These experimental elaborations are regarded as a perfect paradigm in which molecular mechanisms of physiopathology in human diseases and mice models are reciprocally validated.

Gastroenterological tumor

Alimentary canal epithelial tumors with aberrant TGF- β signaling usually emerge as part of the JPS or in the form of sporadic gastric, intestinal, and colorectal adenocarcinomas [39-42]. LOH at 18q, homozygous deletion or intragenic mutations of SMAD4 gene [3,16] as well as promoter hypermethylation [43,44] are widely observed in sporadic gastroenterological tumors. JPS is a rare autosomal dominant disorder characterized by a predisposition to hamartomatous polyps and cancers of the gastrointestinal and colorectal tract. This syndrome is caused by germline mutation of either SMAD4 (15%-20%) or bone morphogenetic protein receptor type IA (*BMPRI1A*) (20%-25%) [17,45-49]. Supporting evidence for SMAD4 haploinsufficiency in tumor initiation and progression is provided by studies on heterozygous *Smad4^{+/-}* mice. Gastric, duodenal, and colonic polyps morphologically resembling those of human juvenile polyposis develop in all aged *Smad4^{+/-}* heterozygous mice. LOH and malignant transformation are frequently observed at later stages of *Smad4^{+/-}* tumors [50-53]. Until recently, by using a

Sleeping Beauty system to generate transposon-based insertional mutations in the gastrointestinal epithelium of mice, Starr and his colleagues have generated mouse mutants by phenocopying the initiation and progression of human gastrointestinal tumors, and identified driver genes including *adenomatous polyposis coli* (*Apc*), *phosphatase and tensin homolog deleted on chromosome 10* (*Pten*), *Bmpr1a*, and *Smad4* [54].

Lines of evidence indicate that *SMAD4* deficiency not only initiates gastroenterological carcinogenesis, but also functions during progression towards malignancy that commonly requires the compromise of other tumor suppressor pathways. Embryonic liver fodrin (ELF) is a crucial adaptor protein in TGF- β signaling and is required for Smad3 and Smad4 localization and signaling. Significant loss of ELF expression is often coupled with reduced SMAD4 expression in human gastric and colonic cancer tissues [55-57]. Similarly, a spectrum of early-onset gastrointestinal tumors ranging from oral to colonic lineage develops in *elf^{-/-};Smad4^{+/-}* mutant mice, indicating a synergistic role of ELF and Smad4 in tumor suppression [58-60]. APC is a member of the WNT signaling pathway and the most commonly mutated gene in human colorectal cancer [61-63]. The *in cis* compound *Apc^{+/-};Smad4^{+/-}* gastrointestinal polyps develop into more malignant tumors as compared to the tumors in the simple *Smad4^{+/-}* or *Apc^{+/-}* heterozygotes [50,64]. Further studies indicated that the loss of Smad4-mediated TGF- β signaling in tumor epithelial cells induced the accumulation of immature myeloid cells through a CCL9/CCR1 chemotactic loop that promote tumor invasion [65].

Recently, multipotent intestinal stem cells that generate the entire epithelial structure are found to be located at the specific site of villus, and are likely to play the role of "cancer stem cells" during tumorigenesis [66-71]. Since BMP signaling plays an important role in the stem cell renewal function [72,73], it is of great importance to dissect the contribution of intestinal stem cell-specific TGF- β signaling pathway to the gastrointestinal carcinogenesis.

Squamous cell carcinomas in the skin and upper digestive tract

The epidermis of the skin, the mucosa of the oral cavity and esophagus comprise most of the stratified squamous epithelia of the body, and they share common ground on aspects of tissue genesis, differentiation and even oncogenic transformation. TGF- β is an important regulator of squamous epithelial cell development and the maintenance of tissue homeostasis. The biphasic role of TGF- β as both a tumor-suppressor and a tumor-promoter has been

validated in mouse model overexpressing TGF- β 1 in keratinocytes [74]. However, a majority of *in vivo* evidences have supported the concept that TGF- β signaling is primarily a tumor-suppression pathway with growth inhibitory effects. Keratinocytic Smad4 is the major transducer of TGF- β and BMP signaling, both of which exert their unique influences on epidermal biology [75-77]. In human skin squamous cell carcinomas, 57% samples exhibited LOH at the *SMAD4* locus. The incidence of loss of SMAD4 expression was high, particularly in poorly differentiated skin carcinomas [78]. Keratinocyte-specific loss of *Smad4* in mice resulted in spontaneous skin tumor formation at as early as 5 months of age, indicating that *Smad4* deficiency initiated squamous cell carcinoma formation. Notably, Smad4 has been shown to interact with the PTEN/Akt signaling pathway to repress skin tumor formation [79,80]. The synergistic role of Smad4 with PTEN in suppressing epidermal and esophageal tumorigenesis has further been confirmed in keratinocyte-specific *K5-Cre;Smad4^{Co/Co};Pten^{Co/Co}* mice. Smad4 and PTEN have been shown to suppress esophageal tumorigenesis through the cooperative induction of cell cycle inhibitors [81]. On the other hand, enhanced Smad4 binding to the Snail promoter likely contributes to Smad2 loss-associated Snail activation and EMT during skin carcinogenesis [78]. A very recent study has revealed the casual role of Smad4 loss in head and neck squamous cell carcinoma (HNSCC) development and progression. In either human HNSCC or mouse models in which *Smad4* was specifically deleted in the upper digestive tract, Smad4 downregulation occurred at the stage prior to tumor formation. Further analyses suggest that *Smad4* loss causes HNSCC formation and invasion which is largely due to defects in the Fanconi anemia/Brca DNA repair pathway, increased genomic instability and inflammation [82].

A number of studies have recently identified follicle stem cells that reside in a quiescent niche and can give rise to all skin epithelial lineages [83-85]. Our latest work has implicated that loss of Smad4 induces hyperactivation of follicle stem cells which is associated with skin squamous cell carcinoma formation in mice, and eventually results in the depletion of follicle stem cells, thereby indicating that Smad4 plays a pivotal role in follicle stem cell maintenance [86]. Increasing evidences suggest that the harmonization of TGF- β /BMP signaling with other pathways, including Sonic hedgehog, Wnt, Notch, and Akt signaling pathways is required for achieving balanced self-renewal and activation of multipotent follicle stem cells [86-93].

Other cancers

Cancers of the breast, liver, and prostate are among the most prevalent human cancers. However, the role of TGF- β signaling in the initiation and progression of these diseases is not as explicit as in pancreatic and colorectal cancers. Investigators have reported infrequent alteration of the *SMAD4* gene or its protein product in these cancers. For example, the LOH of 18q have been reported in these cancers, but *SMAD4* did not appear to be the target of inactivation [94]. Intragenic mutations of *SMAD4* were also rarely observed, particularly in liver and prostate cancers [95-99]. Most prostate cancers become resistant to the antiproliferative effects of TGF- β without defined mutations or deletions of the members of the Smad signaling pathway [100]. Tissue-specific ablation of *Smad4* in hepatocytes and bile duct epithelial cells causes neither discernable defects on liver development nor tumor formation [101]. *Smad4* deletion in mammary epithelium gradually induced well-differentiated squamous cell carcinomas in all the mutant mice but with a long latency, and enhanced canonical Wnt signaling likely contributes to *Smad4* loss-associated epithelial transdifferentiation during carcinogenesis [102]. These data revealed that, at least in prostatic epithelium and hepatocytes, the absence of *Smad4* alone cannot drive the initiation of tumorigenesis, but may require the participation of other cancer-related genes, for example, a combined loss with *Pten* [101].

Non-cell autonomous effect of *Smad4*-mediated TGF- β signaling in suppressing tumorigenesis

The reasons for non-phenotype of some tissue-specific *Smad4* knockout mice are unclear, but may be interpreted as an outcome in which the malignant phenotype is held in check by the appropriate microenvironment [2]. In addition to genetically damaged cells, tumorigenesis is induced by an in situ tumor-favoring microenvironment normally comprising a complicated network of signals derived from many cell types.

It is being increasingly recognized that the neighboring cells in the microenvironment of the tumor may be the source of mutation, and thus the original cause of the tumor [14]. Compelling evidences have been derived from stromal cell-specific knockout mouse models. Conditional inactivation of the *Tgfb2* gene in mouse fibroblasts unexpectedly resulted in intraepithelial neoplasia in the prostate and invasive squamous cell carcinoma of the forestomach, and both were associated with an increased

abundance of stromal cells [103]. These *Tgfb2*-deficient fibroblasts also promoted growth and invasion of co-transplanted mammary carcinoma cells [104,105]. Disruption of TGF- β signalling in T cells through transgenic expression of a dominant negative *Tgfb2* was shown to accelerate dextran sulfate sodium/azoxymethane-induced colon carcinogenesis [106]. A convincing study reveals that *Smad4*-mediated TGF- β signaling exerts a non-cell autonomous effect in tumorigenesis. Selective loss of *Smad4*-dependent TGF- β signaling in mouse T cells results in spontaneous epithelial cancers throughout the gastrointestinal tract, while no tumorigenesis is observed in 2 mouse models with the deletion of the *Smad4* gene restricted to the epithelial lineage. In addition, all heterozygotes of conditional mice showed a haploinsufficiency for *Smad4* in T cell lineage during tumorigenesis, supporting the hypothesis that a compromised TGF- β signaling in T cell contributes to the etiology of human FJP [107]. Our recent study has also suggested that epithelial tumorigenesis could largely be accounted to the wrong message sent by neighboring mesenchymal cells. Human keratocystic odontogenic tumors (KCOT) are benign uni- or multicystic intraosseous tumours of odontogenic origin with a high recurrence rate as well as a potential for aggressive behavior. Human KCOT usually harbor *PTCH1* or *PTCH2* mutations in the tumor squamous epithelium. However, odontoblast-specific *Smad4* knockout mice surprisingly exhibited 100% penetrance of odontogenic keratocysts resembling human KCOTs. The integrity of *Smad4* remains unchanged within the KCOT entities. Further analysis revealed that the deletion of *Smad4* in odontoblasts, which changed the fate of odontoblasts, could also alter the fate of the neighboring Hertwig's epithelial root sheath (HERS) and epithelial rests of Malassez (ERM), which are genetically normal, thus leading to the formation of KCOT [108]. In these knockout models, tumor-promotion effects are mediated by the alteration of paracrine signals released by genetically manipulated cells into the microenvironment. Reduced expression of BMPs by *Smad4*-deficient odontoblasts may account for the ceaseless expansion of ERMs. Similarly, KCOTs that emerge from *Smad4*-deficient ERMs, which fail to receive TGF- β /BMP signals from odontoblasts, are frequently observed in the keratinocyte-specific *Smad4* knockout mice [108]. Therefore, the aforementioned mouse models have introduced a heuristic notion that in addition to the accumulation of somatic mutations in epithelial cells, genetic defects in stromal cells also contribute considerably to the development of epithelial tumors.

Conclusion and perspective

In vivo studies have revealed important physiological functions of Smad4-mediated TGF- β signaling in suppressing tumorigenesis via either cell-autonomous or non-cell autonomous mechanism. However, different experiments have apparently conflicting conclusions. These may largely be due to the fact that TGF- β has numerous and opposite effects on cells and the surrounding microenvironment; Smad4-mediated cellular responses to TGF- β signaling vary with extracellular matrix, ligand concentration, and cell type specific cofactors at different developmental stages. Investigating new components that could modify the Smad4-mediated TGF- β pathways and new targets that participate in the context-dependent effects of TGF- β signaling constitute the next step of the challenge. Increasing data have implicated that micro RNAs (miRNAs) play roles in TGF- β /Smad-pathway-induced tumor-suppressive effects [109,110]. Undoubtedly, miRNAs will be receiving more attention as the components of the TGF- β signaling pathway, and these might facilitate comprehensive understanding of the mechanisms underlying the function of TGF- β signaling in the suppression of tumorigenesis. Better understanding of the precise mechanisms that enable TGF- β and their downstream effectors to function in different cell types may facilitate the development of successful therapies targeting TGF- β signaling in the fight against cancers.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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